Bioaccumulation and Effects of 17β-estradiol in Male Goldfish (Carassius auratus): Influence of Carbon Nanotubes

Zhen-Hua YAN1,* and Hong-Wei SUN1

1Key Laboratory of Integrated Regulation and Resource Development on Shallow Lakes of Ministry of Education, College of Environment, Hohai University, Nanjing 210098, China
*Corresponding author

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Abstract. More attention have been paid to the increasing release of multiwall carbon nanotubes (MWCNTs) into aquatic environments, which may interact with the co-existed contaminants and further alter their bioactivity. In this study, we investigate the influence of MWCNTs on the effects of 17β-estradiol (E2) in male goldfish (Carassius auratus). Compared with E2 alone exposure, the bioaccumulation of E2 in the liver were markedly reduced to the baseline by the presence of MWCNTs, as well as in the estrogenic responses (vtg and esr1 gene) in a concentration dependent manner. The presence of bile salts did not alter the release of estrogen from MWCNTs when compared to the control. These findings indicate that MWCNTs protect fish from threat by estrogen through strong adsorption.

Introduction

In the last two decades, the residues of estrogens in aquatic environments have attracted many attention because their serious threat to the health of animals and humans. These compounds from point and non-point sources are continuously released into the environment, and have been detected in wastewater, surface water, groundwater, and even drinking water. Although the detected concentrations are always at ng/L levels, it is sufficient to induce some of the estrogenic effects on fish, such as vitellogenesis, hermaphrodite, and even population collapse [1].

Among these estrogens, 17β-estradiol (E2) is a representative compound because of its natural origin and high estrogenic potency. Every day, human females will excrete 5 µg of E2 through urine, which will reach to 100 µg/day in the menstrual period. These excretion are account for 50% of the natural estrogens in wastewater [2]. Given that the traditional processes in wastewater treatment plants are insufficient to completely degrade this estrogen, E2 could directly discharge into surface water. For instance in Taihu Lake, E2 was also regarded as the major estrogenic contributor, which induced at least 50% of the overall estrogenic activities in the lake water [3]. In addition, E2 was also included in the watch list under the European Water Framework Directive due to its potentially harmful effect on ecosystem [2]. Hence, it is necessary to effectively remove this estrogen from waters with several advanced processes, such as adsorption with carbon nanotubes (CNTs).

Due to the strong adsorption property and extremely small size, CNTs have been widely applied in composites, medicine, and environmental modification. In the past 2016, the market of CNTs-based composites has reach to 50,000 tons and $384 million [4]. Inevitably, CNTs would be released into aquatic environments and adsorb other
co-existed contaminants (e.g. E2), changing their fate, bioavailability, and bioactivity. Given that the concentrations of contaminants on the surface of CNTs were higher than that in the surrounding environment, the intake of a minor amount of CNTs by organisms could bring abundant CNT-associated contaminants into the organisms, resulting an unpredictable risk [5]. Recently, several studies have been conducted to understand the influence of CNTs on the bioactivity of contaminants in non-target organisms, however, results are often inconsistent.

In this study, the influence of MWCNTs on the effects of E2 in male goldfish (Carassius auratus) was investigated. The concentrations of E2 accumulated in the liver were detected after exposure fish to MWCNTs and E2 (alone or in combination) for 5 and 10 days, as well as the gene expression of vtg and esr1 involved in estrogenic responses. The release of E2 from MWCNTs was simultaneously conducted in bile salts to understand the influence of biofluids on the interaction between MWCNTs and E2 in fish.

Material and Methods

Chemicals and Reagents

E2 (>98% purity) were purchased from Sigma-Aldrich (St. Louis, USA). Cleaned MWCNTs power were obtained from XFNANO Materials (Nanjing, China), with the purity >99.9%, inner and outer diameter at 8 nm and 3 nm, and length at 0.5-2 µm. MS-222 and DMSO was purchased from J&K Chemical (Shanghai, China).

Stock solutions of E2 were prepared in DMSO, and further diluted by test water to the working concentrations, with the final DMSO concentration in each treatment <0.01%. The MWCNTs were prepared in ultrapure water with sonication for 2 h at 25 °C with an output of 100 W and 40 kHz. And then, it was also diluted with test water by sonication. Before the experiment, all solutions were equilibrated for 24 h.

Animal Culture and Exposure Experiment

Male goldfish (17.6 ± 1.5 g, 12± 1.6 cm) were obtained from the Nanjing Institute of Fishery Science (Nanjing, China) and acclimatized for two weeks. After that, fish were randomly distributed into different treatments for 5 and 10 days, including control (C), MWCNTs alone (M1: 0.1 mg/L, M2: 1 mg/L, and M3: 10 mg/L), E2 alone (E: 100 ng/L), and their mixtures with the corresponding concentrations (E+M1, E+M2, and E+M3), respectively. Three replicates were used for each treatment, and half of the test solution was renewed daily. The E2 concentration in this study was environmentally relevant since it was lower than the maximal concentrations detected in the Venice lagoon [6].

After exposure for 5 and 10 days, twelve fish from each treatment were collected and anaesthetized with MS-222. And then, liver were sampled after fish were sacrificed, and immediately stored in liquid nitrogen. A part of the liver was treated with RNAiso Reagent (TaKaRa, Japan) and RNase-free DNase I (Fermentas, Canada) to gain pure total RNA used for gene expression analysis. The other was extracted by pressurized liquid extraction and concentrated for further chemical analysis.

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Two important genes involved in estrogenic effects in goldfish was determined as our previous study [7], and the primer sequences are shown in Table 1. The primers were synthesized by Life Technologies (Shanghai, China). Expression of target genes were
quantified by a Bio-Rad CFX96 Touch real time PCR determination system (Bio-Rad, USA). β-Actin was used as a reliable reference gene to normalize the expression of target genes.

Table 1. Quantitative real-time PCR primer sequences. GenBank accession numbers are provided.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequences (5’-3’)</th>
<th>GenBank number</th>
</tr>
</thead>
<tbody>
<tr>
<td>esr1-forward</td>
<td>CCCGCTCTATGACCTTTTG</td>
<td>AY055725</td>
</tr>
<tr>
<td>esr1-reverse</td>
<td>CTGCTGTGTGTGGGTGTA</td>
<td></td>
</tr>
<tr>
<td>vtg-forward</td>
<td>AAGGCCACCATTACACCTC</td>
<td>DQ641252</td>
</tr>
<tr>
<td>vtg-reverse</td>
<td>CTTTCCTCTCGCCATAACA</td>
<td></td>
</tr>
<tr>
<td>β-actin-forward</td>
<td>ATGCGGAAACTGGAAAGGG</td>
<td>AB039726</td>
</tr>
<tr>
<td>β-actin-reverse</td>
<td>GAGGGCAGAGTGTTAGACG</td>
<td></td>
</tr>
</tbody>
</table>

Liver E2 Concentrations

The concentrations of E2 in liver were detected using an ultrahigh-performance liquid chromatography tandem mass spectroscopy (LC-MS/MS) (Agilent Technologies, USA) following our previous study [7]. The E2-d4 was used as internal standard. The limit of detection (LOD) and limit of quantitation (LOQ) of E2 in liver was 0.3 ng/g and 1.0 ng/g, respectively. Its recovery ranged from 85% to 115% in the fish liver.

Statistical Analysis

All statistical analyses were performed in the SPSS. All data were expressed as the mean ± standard deviation. A one-way analysis of variance (ANOVA) with Tukey’s HSD test was used to determine statistical significance and multiple-comparison among different treatments. \(P < 0.05\) were considered to be significant.

Results

Concentrations of E2

There was no mortality or abnormal behavior of fish during the exposure periods. In the liver of male goldfish, very little E2 was detected in the control livers, as well as the liver of MWCNTs alone treatments (Fig. 1). Exposure to E2 alone induced a 16 to 19-fold increase in the bioaccumulation of E2. However, this bioaccumulation was markedly decreased by the co-existed MWCNTs in a concentration dependent manner, with a decrease ranged from 64%-99%.

Figure 1. Concentration of E2 in the liver of male goldfish in different treatments. Bars not sharing common letters (a-d) were significantly different from each other (\(P < 0.05\)).
**Estrogenic Response**

A significant increase in the gene expression of *vtg* and *esr1* was measured in the liver of male goldfish after exposure to E2 alone when compared to the controls, with no response apparent in fish from the MWCNTs treatments (Fig. 2). Interestingly, these induced gene expression by E2 were further reduced by the presence of MWCNTs in a concentration dependent manner, with a 60%-95% decrease in the *vtg* gene and a 39%-92% decrease in the *esr1* gene, respectively.

![Figure 2. Gene expression of vtg (A) and esr1 (B) in the liver of male goldfish in different treatments. Bars not sharing common letters (a-f) were significantly different from each other (P < 0.05).](image)

**Discussions**

The presence of MWCNTs significantly inhibited the accumulation of E2 in the liver, as well as the related gene expression of estrogenic response, suggesting a protective effect of MWCNTs in a concentration dependent manner. These inhibitive effects may mainly attribute to the strong adsorption of E2 on MWCNTs, which would lower the bioavailability of the adsorbed E2 in organisms. Similar to our results, the effects of 17α-ethinylestradiol (EE2) in zebrafish (*Danio rerio*) were significantly reduced and even eliminated by the coexistence of fullerene in waters because of the strong absorption within fullerene aggregates [8]. Compared with the perfluorooctane sulfonate (PFOS) alone exposure, the adverse effects induced by PFOS on the hatching rate, heart rate, and body length of zebrafish larvae were significantly alleviated in the presence of MWCNTs, as well as the mortality and malformation. The adsorption of PFOS to MWCNTs may be responsible for this antagonism. In the sediment, the retention of flame retardants seemed to be favored by fullerene presence, which would minimize their impact on living organisms [9]. In the root of collard greens (*Brassica oleracea*), the co-exposure of carbon materials notably suppressed the carbamazepine accumulation, with a suppressed rate of 29% to 89% by different carbon materials [10]. The adsorption capacity of the carbon materials correlated well with the suppression of carbamazepine accumulation.

However, Bisesi et al. [11] reported that the presence of single-walled carbon nanotubes (SWCNTs) did not alter the extent of EE2-driven induction of *vtg in vivo* compared to the levels in organisms treated with EE2 alone. The adsorbed EE2 on SWCNTs may desorb and retain bioactivity under biological conditions. While CNTs were ingested by organisms, the presence of biomolecules in the body may significantly influence the release of bound contaminants on CNTs, therefore inducing unexpected toxicity. In our previous study, the presence of bile salts resulted in a higher desorption of roxithromycin (ROX) when compared to that in the background solution. Competition with contaminants for the same adsorption sites on MWCNTs by
biomolecules would be primarily responsible for the increased release of ROX from MWCNTs [12]. In this study, the release of E2 from MWCNTs was also investigated in the presence of bile salts following our previous study [12]. The results showed that less than 5% of the adsorbed E2 released from MWCNTs into waters, and the presence of bile salts at this concentration had no effect on the desorption of E2 from MWCNTs, suggesting a highly stable adsorption of E2 on MWCNTs. In consistent with this result, Song et al. [5] also demonstrated that the sorption of EE2 on SWCNTs in the presence and absence of biomolecules was hardly reversible, and less than 0.2% of the total adsorbed EE2 were released.

Generally, the presence of MWCNTs significantly reduced the E2 induced accumulation and estrogenic responses in male goldfish in a concentration dependent manner, showing an inhibitive effect. This may be mainly attributed to the stable adsorption of E2 on MWCNTs even in the gastrointestinal conditions. More attention should pay to the presence of nanomaterials in the environment since these materials may interact with the coexisted contaminants and further result in an unexpected risk.

Acknowledgement

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